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How female reed buntings benefit from extra-pair mating behaviour: testing hypotheses through patterns of paternity in sequential broods

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Abstract

Extra-pair paternity is an important aspect of reproductive strategies in many species of birds. Given that in most species females control whether fertilization occurs, they are expected to benefit in some way from the extra-pair matings. In this study we use patterns of extra-pair paternity (EPP) in broods of individual reed buntings (*Emberiza schoeniclus*), both within and between seasons, to test four hypothesized female benefits: (1) assessing potential future partners and seeking (2) genetic diversity (3) good genes, or (4) compatible genes. Reed buntings are socially monogamous, multibrooded passerines with extremely high levels of extra-pair paternity. We studied a population of reed buntings in the Netherlands in 2002 and 2003; 51% of offspring in 74% of nests were extra-pair. We showed that patterns of EPP did not support the first and second hypotheses, since females did not form a pair with previous extra-pair partners, EPP was not evenly distributed among broods and more broods than expected were sired by a single male. Furthermore, there was no relation between a male's within- and extra-pair fertilization success, no consistency in EPP between breeding attempts, no effect of parental relatedness on EPP and several cases of reciprocal paternity. These patterns do not support the good genes hypothesis and are most consistent with the genetic compatibility hypothesis. However, our previous finding that older males are more successful in gaining EPP, suggests some effect of good genes. These hypotheses need not be mutually exclusive, as females may select compatible males above a certain quality threshold (e.g. old males).

Keywords: *Emberiza schoeniclus*, extra-pair paternity, genetic benefits, genetic compatibility, good genes, mate switching

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Introduction

Social monogamy, where a male and female form a pair and collaborate in raising offspring, is the most common mating system in birds (Lack 1968). However, recently molecular techniques have revealed that genetic monogamy is rare, as it is found in only 14% of social monogamous passerine species studied so far (Griffith *et al*. 2002). Males may gain direct benefits by engaging in extra-pair copulations (EPCs), as these can lead to extra-pair fertilizations (EPFs), increasing a male's reproductive output without additional paternal investment (Westneat *et al*. 1990; Birkhead & Møller 1992). Since the maximum reproductive success for females is limited by the number of offspring they can raise and the trade offs against survival, the benefits to females of EPCs are less clear. Given that females have at least some control over whether insemination occurs and EPCs are likely to be costly (Birkhead & Møller 1992), females are only expected to engage in EPCs if there are potential benefits. Females may gain direct benefits, such as fertility insurance (Wetton & Parkin 1991), foraging rights on the extra-pair male's territory (Gray 1997) or the opportunity to assess males as potential future partners (Heg *et al*. 1993; Cézilly & Nager 1995). On the other hand, females may gain potential indirect benefits such as increasing the genetic diversity (Brooker *et al*. 1990), the absolute quality ('good genes', e.g. Kempenaers *et al*. 1992; Hasselquist *et al*.

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1996; Kempenaers *et al*. 1997; Sheldon *et al*. 1997; Richardson & Burke 1999), or the relative quality ('genetic compatibility'; Johnsen *et al*. 2000; Tregenza & Wedell 2000; Foerster *et al*. 2003; reviewed in Jennions & Petrie 2000; Eimes *et al*. 2005; Tarvin *et al*. 2005) of their offspring. However, evidence is accumulating that these findings in support of genetic benefit hypotheses cannot simply be generalized, since several studies report no fitness differences between maternal half-siblings (Schmoll *et al*. 2003; Kleven & Lifjeld 2004).

One way to investigate the validity of these hypotheses is to determine patterns of extra-pair paternity (EPP) in a series of broods produced by the same pair. This approach enables us to test between predictions that are specific to four of the hypotheses mentioned above. First, if females aim to increase the genetic diversity of their offspring, all females are expected to engage in EPCs; therefore most or all broods are expected to contain extra-pair young (EPY) that are sired by a number of different males (Westneat *et al*. 1990). Second, if females seek good genes for their offspring, only females paired with a low quality male (defined as a male with few extra-pair young (EPY)) should engage in EPCs; broods are expected to be sired by a single, preferred, male (Westneat *et al*. 1990) and paternity in sequential broods of the same pair is expected to be consistent (females being either faithful or unfaithful). As high quality males are expected to be preferred both by their own females as well as by extra-pair females, we expect a negative relationship between the number of EPY a male gains in a season, and the percentage of paternity he loses in his own nests (Kempenaers & Dhondt 1993). Females are expected to choose the same extra-pair male for the next breeding attempt, if he is still alive (Weatherhead 1999). Third, if females seek compatible genes for their offspring, only females paired to a genetically incompatible male should engage in EPCs and broods are expected to be sired by the most compatible male, which is similar to the expectations of the good genes hypothesis. However, the compatibility of members of a pair depends on both partners, therefore certain males are not expected to be preferred by both their own females as well as extra-pair females. Thus in contrast to the good genes hypothesis, the genetic compatibility hypothesis allows reciprocal EPP between males and there is likely to be less variability in male mating success (Kempenaers & Dhondt 1993). Fourth, if females use EPCs to assess future mates, females with EPP in the first nest are expected to be more likely to change social mates before the next breeding attempt in the same or the following season than faithful females, and possibly select the former extra-pair mate as the next social partner (Heg *et al*. 1993). Several studies have examined variation in paternity for the same individuals between breeding seasons (e.g. Dunn *et al*. 1994; Yezerinac *et al*. 1996; Kempenaers *et al*. 1997; Perreault *et al*. 1997; Lubjuhn *et al*. 1999; Weatherhead 1999; Friedl & Klump 2002; Dietrich *et al*. 2004), and among broods within years (double-brooded species, e.g. Dixon *et al*. 1994; Stutchbury *et al*. 1994; Freeman-Gallant 1996; Yezerinac *et al*. 1996; Conrad *et al*. 1998; Lubjuhn *et al*. 2001; Peterson *et al*. 2001; Beheler & Rhodes 2003; Dietrich *et al*. 2004). However, none of these studies used patterns of paternity to their full extent in distinguishing between these female benefit hypotheses.

We studied patterns of paternity in a population of reed buntings (*Emberiza schoeniclus*) in the Netherlands. The reed bunting is a small (18 g), sexually dimorphic passerine. Social monogamy is the most common mating system, with high levels of EPP (50% of offspring in 80% of nests; Bouwman *et al*. 2005). This species is capable of raising two successful broods in a single season. Adults show high site fidelity between breeding seasons (O'Malley 1993), thereby presenting an ideal opportunity to study patterns of EPP within individuals, both within and between seasons. In this study we investigate whether patterns of EPP in sequential nests of the same individuals match the predictions that arise from the four hypotheses to explain why females may benefit from engaging in EPCs.

Materials and methods

Data collection

In 2002 and 2003 a population of reed buntings was studied in a 13 ha study site, on the island of Noorderplaat (45 ha) in the De Biesbosch National Park in the Netherlands (51°45′N, 4°45′E). The vegetation consisted of a combination of reeds (*Phragmites australis*), soft rush (*Juncus effusus*), hard rush (*Juncus inflexus*) and various species of grasses. The height of the vegetation varied from 50 to 300 cm, with most of the vegetation below 150 cm. A grid with cells of approximately 20×40 m was laid across the area for mapping territories and nests, using two-metre high bamboo poles (individually marked with coloured tape) placed at every intersection (Fig. 1).

Males arrived before females on the breeding site starting from the end of February, and occupied a territory. Pair formation occurred one to two months before the onset of breeding. Territories were mapped by plotting the location of the singing posts of males. No territorial conflicts were observed; therefore strict boundaries could not be drawn between neighbouring territories. In 2002 and 2003, respectively, 44 and 35 males held a territory in our study area. During the breeding season we did not catch or see any adult males that did not defend a territory (i.e. floating males), despite being in the study site on a daily basis throughout the breeding season and catching regularly (i.e. on average every three days, for approximately four hours a day, using 100–150 m of mist nets).

Within our study site, 96% ($n = 158$) of adult reed buntings were caught using mist nets. The majority of individuals

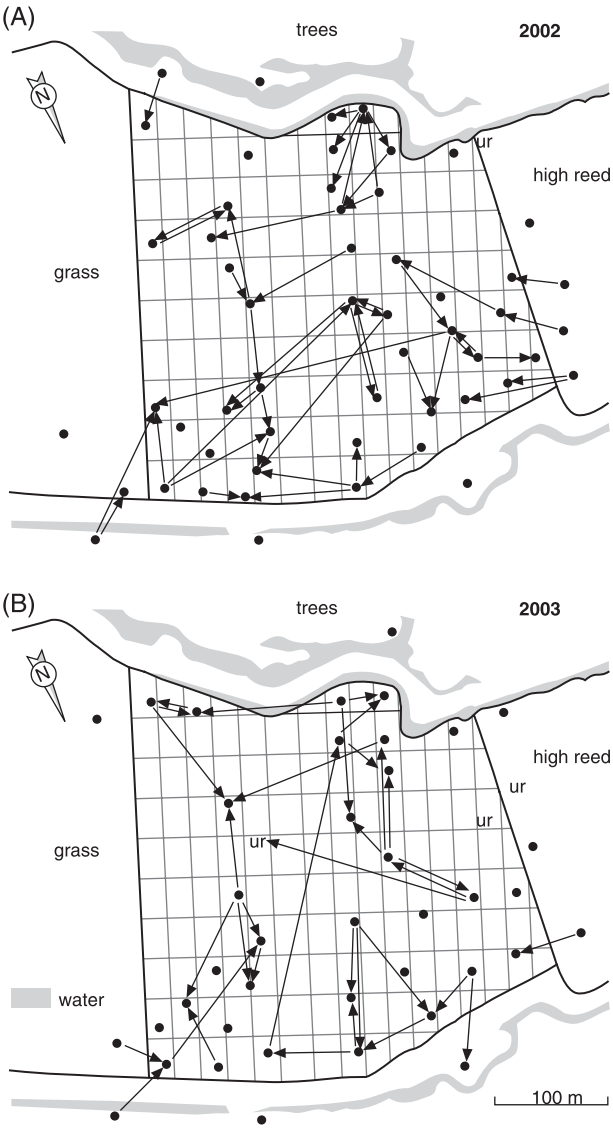

Fig. 1 Map showing the extra-pair mating behaviour in (A) 2002 and (B) 2003. The principal song-post of each ringed male, as an indicator of the centre of a territory, is identified by a spot. Unringed males are identified by 'ur'. The grid used for locating territories is shown; every section is approximately 20 by 40 m. Arrows originate in the territory of the extra-pair male, and point at the territory of the cuckolded male.

(males: 80%; $n = 79$; females: 54%, $n = 79$) were caught between 15 February and 15 April, which marks the approximate beginning of the fertile period of females (i.e. one week before the first egg of the season is laid; date of first egg: 2002: 22 April; 2003: 28 April). Birds were ringed with a numbered aluminium ring and a specific combination of three colour rings, one above the aluminium ring and two on the other leg, for individual recognition. A blood sample (20 μL) was taken from the brachial vein and stored in 96% ethanol at room temperature. The identities of the male and female belonging to a nest (territorial birds) were determined by direct or video observations of colour-ringed birds protecting the nest, incubating and feeding nestlings (for a description of the method using video recordings see Bouwman *et al*. 2005).

Nests are built on or just above the ground and are only used for a single nesting attempt. Clutches consisted of 2–6 eggs (4.16 ± 0.13, $n = 144$). Nests were located through systematic searches that flushed females off the nest, or through observing territorial birds for any nest-related activities. Nestlings were blood-sampled two days after hatching by taking a small blood sample (10 μL) from the leg vein. If eggs did not hatch, we inspected them for embryonic development which, if present, was used as a source of DNA. Within our study site we found 97.4% of all nests that fledged young ($n = 78$); only in two cases did we see fledglings without locating the nest. However, due to high levels of predation (see below); we were unable to locate all nests in the study area. As there was no obvious difference in risk of predation across the site, we believe we obtained a random sample of individual reproductive success for all individuals in our site.

To increase the number of DNA samples from sequential nests, we induced renesting by removing the first clutches of 17 pairs after six to 11 days of incubation (2002: 6 pairs, 2003: 11 pairs; total incubation period: 12–14 days; under licence of the Dutch ethical committee). The embryos of the first clutch were then used as a source of DNA, while the replacement clutch was blood-sampled after hatching. There was no difference in the percentage of females that laid a replacement clutch following natural predation during the incubation stage (80% ($n = 10$)) or following removal of their clutch (82% ($n = 17$); Fisher exact test: $P = 1.0$). Neither was there a difference in the number of days before a replacement clutch was laid following natural predation (median = 5 days, range = 3–29 days, $n = 8$) or clutch removal (median = 5 days, range = 3–14 days, $n = 14$). We thus assume that females whose clutch we removed the clutch behaved as if their nest had been predated.

As was also found in a previous study of reed buntings (O'Malley 1993), there was a high probability of predation at both the egg and nestling stage (67%, $n = 46$ nests; sampled in 2001 without nest protection). The main predators were stoats, *Mustela erminea*, and polecats, *Mustela putorius*; no avian predators were seen in the study area. Therefore in the 2002 and 2003 seasons, after clutch completion a nest was protected against predators using exclosures, of 30 cm height and a diameter of approximately 1 metre, made of wire netting and bamboo sticks and pinned down with tent pegs. Adults were accustomed to the exclosure by putting it around the nest but lowering it to the ground, initially enabling them to walk to their nest. After an hour we checked if the adults had returned to their eggs, indicating they had accepted the presence of the exclosure. If so, we increased the height of the exclosure in 4 steps

(10 cm per 1–2 h), allowing the adults to adjust their flyway into the nest. If the eggs were found to be cold, we removed the exclosure and repeated this procedure two days later. It is possible that the female abandoned her clutch in a maximum of 3 out of 83 nesting attempts due to the exclosure trials, but as none were resighted in the area subsequently these females may themselves have been predated. Three birds did not accept the exclosure after three attempts, after which we left these nests unprotected. To further minimize the risk of predation nest visits were kept to a minimum. Nest protection was removed when the nestlings were approximately 5 days of age. In 2002 the nest protection was found to be very effective, as 93% of broods were not predated when protected ($n = 42$). Apparently predators learned to circumvent the nest protection, as the three nests that were predated were among the last nests of the season. In 2003 broods were occasionally predated early in the season; after 37% of the protected nests had been predated ($n = 27$), we stopped protecting the nests (halfway through the breeding season).

Paternity analysis

DNA was extracted from blood and tissue samples using salt extraction (Richardson *et al*. 2001). The paternity of the nestlings was analyzed using six fluorescently labelled microsatellite markers: *Esc*μ1, *Esc*μ4, *Esc*μ6 (Hanotte *et al*. 1994), *Pdo*μ5 (Griffith *et al*. 1999), *Mcy*μ4 (Double *et al*. 1997) and *Ppi*2 (Martinez *et al*. 1999). PCR amplifications were performed using a Thermolyne amplitron II or a Corbett Research thermal cycler using an initial hot start for 3 min at 94 °C, followed by 30 cycles of 1 minute at 94 °C, 1 minute at annealing temperature and 1 minute at 72 °C. Annealing temperatures were set at 55 °C for *Esc*μ1, *Esc*μ4 and *Mcy*μ4, at 52 °C for *Esc*μ6, at 50 °C for *Pdo*μ5 and at 53 °C for *Ppi*2. Each 10 μL mix contained 10–50 ng of DNA, 1.0 μM of each primer, 0.2 mM of each dNTP, 0.05 units of *Taq* polymerase (Advanced Biotechnologies) and 0.625 mM $MgCl_2$ in the supplied reaction buffer (final concentration 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCL, pH 9.0, 0.01% (w/v) Tween). PCR-products (except for *Ppi*2) were diluted by adding one volume of H_2O. Diluted PCR-products were multiplexed in different combinations. *Esc*μ1, *Esc*μ4 and *Pdo*μ5 were multiplexed in a ratio of 2 : 1 : 2 and *Esc*μ6, *Mcy*μ4 and *Ppi*2 in a ratio of 1 : 1 : 2 for samples of 2002; *Esc*μ1, *Mcy*μ4 and *Ppi*2 were multiplexed in a ratio of 1 : 2 : 2 and *Esc*μ4, *Esc*μ6 and *Pdo*μ5 in a ratio of 2 : 2 : 1 for samples of 2003. One microlitre of multiplex-mixture was mixed with 1.5 μL of a loading buffer containing 1.1 μL of deionized formamide, 0.18 μL of blue dextran loading dye and 0.22 μL of internal lane standard (ROX500, Applied Biosystems). These samples were denatured by heating at 94° for 2 min and then placing directly on ice. One microlitre of each sample was electrophoresed using a 10% denaturing polyacrylamide gel on an Applied Biotechnologies (ABI) 377 XL DNA sequencer. DNA fragments were analysed using DNA fragment analysis software (Applied Biosystems GENESCAN version 3.1 and GENOTYPER version 2.5). Parentage was determined by using a likelihood-based approach in CERVUS (version 2.0; Marshall *et al*. 1998). This program assesses the confidence of paternity assignment using criteria generated through a simulation taking into account allele frequencies in the population, the number of possible candidate parents, the proportion of candidate parents sampled, and the percentage of missing genetic data and genotyping errors. The simulation derives a criterion (the delta value) that estimates the critical difference between the LOD — the natural logarithm of the likelihood ratio-scores of the first and second most likely candidate parents at a level of > 95% confidence and > 80% confidence. Since the method used by CERVUS is based on probability, it can occasionally lead to strange results. Therefore, we also report the number of mismatches between the genotypes of the offspring and those of the putative parents, and between the genotypes of the extra-pair offspring and those of the assigned extra-pair sire. Generally, the genotype of the offspring has a perfect match with the genotype of the real parent, and the occurrence of mismatches indicates mismatched parentage. However, a mismatch between an offspring and its true parent may also arise from genotyping errors or mutations, of which the latter are normally uncommon (Ellegren 2000). Based on the mismatches between the genotypes of offspring and known parents (usually the mother), CERVUS calculates a mean observed error rate across loci. In our dataset CERVUS estimated this error rate to be 1%, indicating that mismatches between offspring and their true parents may occur at a low rate.

In the parentage analysis, we first assessed whether the female and male observed at the nest were the actual parents (using 'exclusion analysis'). First, the maternity of the territorial female was assessed. Then the paternity of the territorial male was assessed using the mother as 'known parent' in the analysis. Using a 'known parent' increases the confidence level when determining the second parent (in this case the father). CERVUS was given the choice between two candidate parents: the territorial female or male and one potential, but unsampled, other female or male. The delta values for the exclusion analysis were calculated by entering the following simulation parameters in CERVUS: 10 000 cycles, two candidate parents present and 50% of candidate parents sampled. Genotypes were available for 99% of all loci. Although the error rate in our dataset was estimated to be 1%, we entered an error rate of 0.01% in the simulation settings of CERVUS. This way the tolerance of mismatches was set to accept up to one mismatch. When using an error rate of 1%, CERVUS accepted up to four mismatches.

Next, paternity was assigned to offspring with genotypes that were not a perfect match to that of the territorial males,

and therefore were possibly sired by an extra-pair male. The genotype of every offspring was set against the genotypes of all ringed males present in the study site in that year (so called 'open analysis'), again using the mother as 'known parent'. The critical values were calculated by entering the following simulation parameters in CERVUS: 10 000 cycles, 68 (2002) or 62 (2003) candidate parents present, 90% of candidate parents sampled, 99% of loci typed, and 0.01% of loci mistyped. If the genotype of the offspring only showed one mismatch with that of the territorial male, and no other extra-pair male provided a perfect match, we assumed that the territorial male was most likely to be the true sire. In 11 cases (2%) an assignment to an extra-pair male was 'forced', when all the following requirements were fulfilled. If a specific male was not ranked as the best candidate by CERVUS, but that male was an extra-pair male which had already sired other offspring in that particular nest, and there were no mismatches between the genotype of the offspring and that male, and the first ranked male did not father any other offspring in that nest, then we decided to accept the specific male as the genetic father.

None of the loci deviated significantly from Hardy–Weinberg equilibrium when including the genotypes of all breeding adults in the analysis. Using the observed allele frequencies, we used CERVUS to calculate a total exclusionary power for the six microsatellite loci; in both years the probability of exclusion was 0.993 for the first parent and 0.999 for the second parent. As a measure for genetic similarity between individuals, we calculated pairwise relatedness (Queller & Goodnight 1989) using the software program KINSHIP 1.2 (http://www.gsoftnet.us/GSoft.html). If more than one extra-pair male sired offspring in a nest, we averaged the female's relatedness to these males.

Data analyses

Unless it is specifically stated that all nests were used, only nests from ringed males containing more than one offspring were included in the analyses, to avoid over-estimating the number of nests with no or all EPY. To avoid pseudoreplication, only one randomly selected nest for each pair was included where appropriate. Statistical analyses were performed using SPSS 11.0.1 (2001). Unless stated, non-parametric tests were used for data that were not normally distributed. Means are expressed with standard errors, probability values are two-tailed and the level of significance was set at $P < 0.05$. To control for the chance of making type I errors (i.e. false positives) when performing multiple statistical tests, it has recently been advocated to control the false discovery rate ('FDR'), rather than using (sequential) Bonferroni corrections (Garcia 2004; Verhoeven *et al*. 2005). When controlling FDR, one controls the proportion of significant results that are in fact type I errors, instead of controlling the chance of making even a single type I error. The advantage of using FDR over Bonferroni corrections is that it is less restrictive and more powerful. Therefore, following the performance of repeated tests in this study, we corrected the significance level α using 'step-up FDR' (Benjamini & Hochberg 1995; Garcia 2004; Verhoeven *et al*. 2005). In the figures we presented the original P values that result from the statistical tests, whereas in the text we presented the corrected α to control for the proportion of type I errors among significant results.

If a brood contained offspring sired by more than one extra-pair male, then both males were included when analysing the mean distance between the cuckolded male's territory and the extra-pair male. When determining the total number of EPFs that a male gained, the analysis was performed both including all males in the study site, and excluding the males from territories on the edge of the study site (outer territories). This was done in order to avoid underestimating the number of EPFs that the peripheral males gained, as we did not sample nests outside our study site.

We tested whether the distribution of EPP over broods differed from what is expected under the binomial distribution using a chi-square goodness-of-fit test. The number of broods expected to contain extra-pair young based on binomial distributions is calculated using the following formula: expected broods = ${}_nC_X * p^{X.} * q^{n-X} * N$, where ${}_nC_X$ (i.e. binomial coefficient) = $n!/[(n-X)!X!]$, p = proportion of EPY in the population, $q = 1 - p$, n = brood size, X = number of EPY per brood, and N = number of broods of size n (Sokal & Rohlf 1994; Perreault *et al*. 1997).

Results

Paternity assignment

In two years, 501 offspring were typed from 129 nests (2002: 280 offspring from 71 nests, 2003: 221 offspring from 58 nests). In 88% of these cases both the territorial male and female were known (2002: 61/71 nests, 2003: 53/58 nests); only the female was known in 5% of nests (2002: 4/71 nests, 2003: 3/58 nests) and only the male was known in 6% of nests (2002: 6/71 nests, 2003: 2/58 nests). Within the nests with a sampled territorial female, 97% of offspring (2002: $n = 254$; 2003: $n = 215$) had genotypes consistent with their being offspring of the female attending the nest at a 95% confidence level, and almost 100% at an 80% confidence level. In total, 99% of offspring were a perfect match with their putative mother ($n = 469$), while the genotypes of five offspring showed one mismatch. No other female provided a better match with these offspring. In one case the genotype of an offspring showed two mismatches, and CERVUS excluded the territorial female to be the mother. This mismatch is expected to be the result of egg dumping (0.2%, $n = 501$ offspring), and the nest was excluded from further analysis.

Paternity was assigned to 87% of offspring (n = 501) with 95% confidence, and to 88% of offspring with 80% confidence. In 217 cases, the offspring showed a perfect match with the male at the nest, while in 18 cases there was one mismatch and in 238 cases there were two or more mismatches between the two. In 15 of the cases were one mismatch was found, no extra-pair male provided a perfect genotypic match, and thus these offspring were considered to be sired by the territorial male. Paternity was assigned with a perfect match to 187 extra-pair offspring, while the paternity of 12 extra-pair offspring was assigned allowing one mismatch. In all these latter 12 cases the genotype of the extra-pair male also showed a perfect match with the genotype of at least one other offspring in the brood. An extra-pair sire could not be assigned to 49 young (10%). On average, 51% of all young were extra-pair (2002: 143/262, 2003: 98/211) and 74% of all nests contained at least one EPY (2002: 55/66, 2003: 34/55; $\chi^2 = 7.14$, d.f. = 1, $P = 0.008$). There was no difference in the average percentage of EPP in nests with at least one extra-pair young between the years (2002 vs. 2003: 65% ± 4% vs. 72% ± 5%; $U = 799.0$, $P = 0.24$). However, since the frequency of nests containing EPP differed significantly between years, we addressed the two years both together and separately in subsequent analyses.

Distribution of EPP

On average, 81% of females produced at least one EPY in a year (2002: 35/39; 2003: 23/33; $\chi^2 = 4.59$, d.f. = 1, $P = 0.03$) and 70% of all EPFs occurred between males and females from neighbouring territories (Figs 1 and 2A). In total, six exchanges in paternity were observed between two males (2002: n = 4; 2003: n = 2). EPY were not evenly distributed among broods (Fig. 2B); the distribution of EPP among broods differed from what is expected under a binomial distribution (Table 1). Broods were sired by one to four different males (Fig. 2C). Out of 78 broods, 29 were sired by a single male, either the social or an extra-pair partner (2002: 16% by social male, 25% by extra-pair male, n = 44; 2003: 41% by social male, 21% by extra-pair male, n = 34). The number of broods sired by a single male was significantly larger than the combined number of broods expected to have either none or all EPY (i.e. thus potentially sired by a single male) from a binomial distribution (2002: $\chi^2 = 17.68$, d.f. = 1, n = 44, $P < 0.001$; 2003: $\chi^2 = 46.51$, d.f. = 1, n = 34, $P < 0.001$; 2002 + 2003: $\chi^2 = 67.38$, d.f. = 1, n = 68, $P < 0.001$).

Consistency of EPP within individuals

We found no general increase or decrease in EPP with time of season (2002: Spearman's rho (r_s) = –0.27, n = 38, $P = 0.10$; 2003: $r_s = 0.12$, n = 31, $P = 0.51$; 2002 + 2003: r_s = –0.10, n = 50, $P = 0.50$). Two successive broods were sampled in a single season for 29 different pairs (2002: 18 pairs; 2003: 15 pairs). There was no significant difference in the proportion of renestings which were a result of removal of the first clutch between years (2002: 5/18; 2003: 7/15; $\chi^2 = 1.26$, d.f. = 1, $P = 0.26$). First broods did not differ systematically from second broods in the percentage of EPY in 2002 (52% ± 9 vs. 55% ± 7; Z = –0.18, n = 18, $P = 0.86$) and 2002 and 2003 combined (54% ± 7 vs. 50% ± 6; Z = –0.46, n = 29, $P = 0.65$), while second broods tended to have a lower percentage of EPY than in first broods in 2003 (56% ± 10 vs. 40% ± 9; Z = –1.74, n = 15, $P = 0.082$). Also in 2003, but not in 2002 and in 2002 and 2003 combined, there was a significant correlation between the percentage EPY in the two broods, indicating that pairs with fewer EPY in their first brood, also had fewer EPY in their second brood (Fig. 3). After correcting for FDR, the correlation in 2003 was no longer significant (corrected $\alpha = 0.017$). Given the

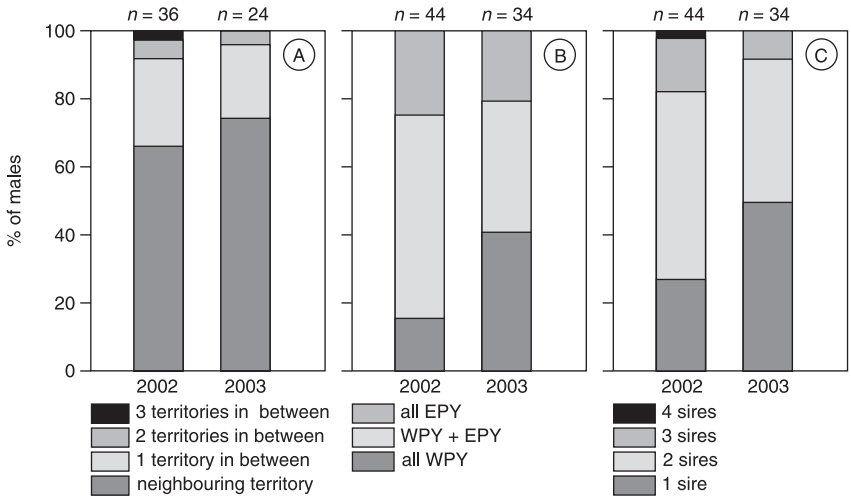

Fig. 2 Percentage of (A) extra-pair fertilizations with 0, 1, 2 or 3 territories between the territories of the cuckolded male and the extra-pair male that gained the fertilization; reed bunting nests with (B) only within-pair young (WPY), only extra-pair young (EPY), or both WPY and EPY, and (C) 1, 2, 3 or 4 males siring one or more offspring in that nest. Sample sizes are shown. After correcting for multiple testing, no significant differences were found between the years for (A) ($\chi^2 = 0.97$, d.f. = 3, $P = 0.81$), (B) ($\chi^2 = 6.38$, d.f. = 2, $P = 0.04$), or (C) ($\chi^2 = 4.89$, d.f. = 3, $P = 0.18$).

Brood size	No. of EPY per brood						Total broods
	0	1	2	3	4	5	
2	2 (1.2)	2 (2.5)	1 (1.3)	—	—	—	5
3	6 (2.0)	3 (6.3)	2 (6.5)	6 (2.3)	—	—	17
4	4 (1.1)	2 (4.6)	4 (7.1)	6 (4.9)	3 (1.3)	—	19
5	7 (0.8)	1 (4.0)	3 (8.3)	6 (8.6)	1 (4.5)	9 (0.9)	27
Total	19 (5.1)	8 (17.3)	10 (23.2)	18 (15.8)	4 (5.8)	9 (0.9)	68

Table 1 Distribution of EPP among nests. The observed and expected (in brackets) values of 2002 and 2003 combined are presented ($P = 0.51$). The distribution of EPP among nests differ from what is expected under the binomial distribution [2002 + 2003: $\chi^2 = 121.62$, d.f. = 5, $n = 68$ nests, $P < 0.0001$; 2002 ($P = 0.55$; data not shown): $\chi^2 = 30.84$, d.f. = 5, $n = 44$ nests, $P < 0.0001$; 2003 ($P = 0.46$; data not shown): $\chi^2 = 86.35$, d.f. = 5, $n = 34$ nests, $P < 0.0001$]

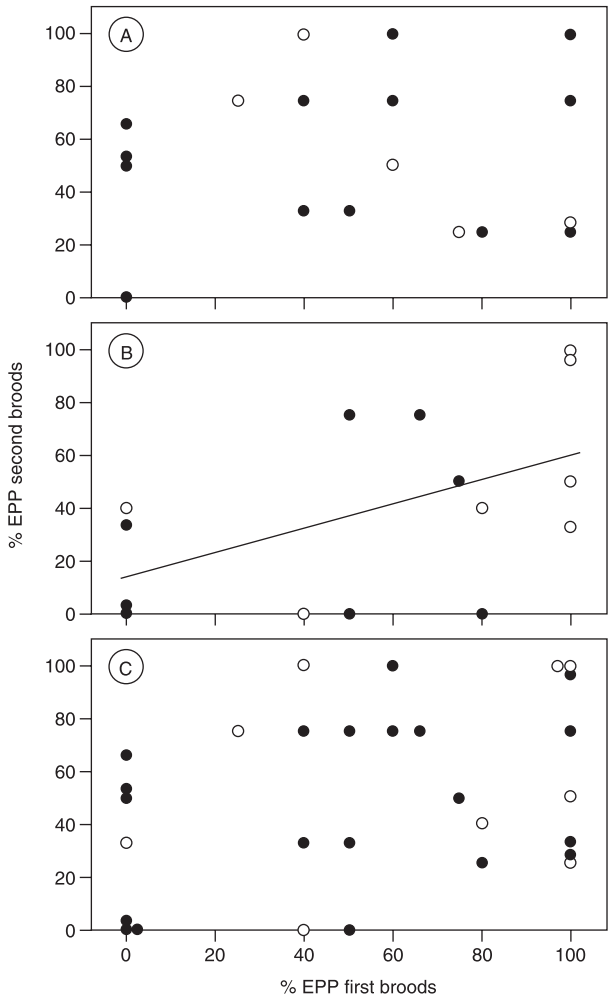

Fig. 3 Consistency in the percentage of EPP of the same pair within a season (A) for 2002 (2002: $r_s = -0.04$, $n = 18$, $P = 0.89$), (B) for 2003 ($r_s = 0.54$, $n = 15$, $P = 0.038$), and (C) for 2002 and 2003 combined ($r_s = 0.30$, $n = 29$ pairs, $P = 0.12$). The pairs for which the first nest was removed are indicated by an open circle.

relatively small brood sizes, the percentage of EPY in a brood can change dramatically if one or a few young are not typed (e.g. due to hatching failure). When only including pairs for which both nests completely hatched, there was no longer a significant correlation between the percentage EPY in both broods, although the sample size has become very small (2002: $r_s = -0.32$, $n = 7$, $P = 0.49$; 2003: $r_s = 0.24$, $n = 4$, $P = 0.76$; 2002 + 2003: $r_s = -0.002$, $n = 11$, $P = 0.99$). Alternatively, if factors influencing extra-pair mating behaviour are similar during the subsequent breeding attempts (e.g. when time between breeding attempts is short), this may lead to comparable levels of EPP in first and second broods. However, we found no difference in the average number of days between subsequent breeding attempts for pairs in 2002 (32.5 ± 2.9 days) and 2003 (31.2 ± 3.6 days; $t = 0.28$, d.f. = 1, $P = 0.78$). When excluding the pairs of which the first nest was removed from the analysis, there was no significant correlation in either of the years (2002: $r_s = 0.26$, $n = 13$, $P = 0.39$; 2003: $r_s = 0.30$, $n = 8$, $P = 0.48$; 2002 + 2003: $r_s = 0.25$, $n = 21$, $P = 0.28$). This may be due to a factor related to the removal of first nests, but it may also be a problem of power since the sample size has been reduced.

While paired to the same social male, 33% (11/33) were 'faithful' to their social male (i.e. no EPY) in their first or second brood, and only 9% (3/33) were 'faithful' to their social male in both broods (Fig. 4). The remaining 67% of females (22/33) were 'unfaithful' (i.e. at least one EPY) in both broods.

Social mate choice

There were 34 pairs of which both members were colour-ringed before the start of the fertile period (i.e. 15 April; 2002: 16 pairs, 2003: 18 pairs). Within these pairs we did not observe any cases of mate switching between the prefertile period and the actual breeding season. In total, 44 females produced more than one clutch within a single season (including all nests found with known territorial male and female; 2002: $n = 21$, 2003: $n = 23$). In three of these cases (all in 2003), the social male disappeared (assumed dead) after the first clutch, and the female remated. Females did not change social partners within the same season when their original social partner was still present in the study site ($n = 41$).

On average, 56% of ringed males ($n = 78$) and 45% of ringed females ($n = 72$) returned the following year. The probability of returning was not influenced by the interaction

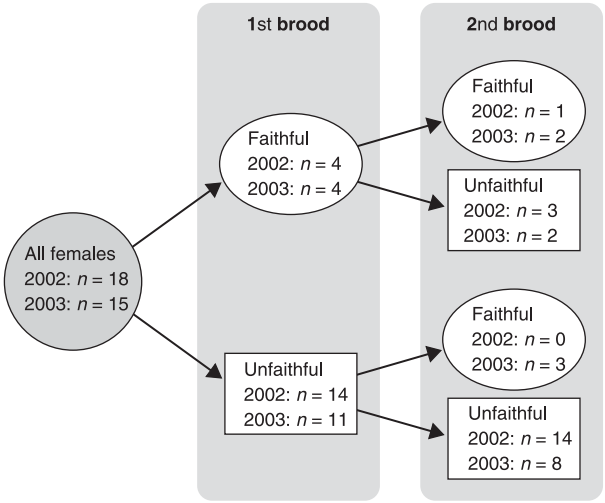

Fig. 4 Consistency in the presence of EPY within subsequent broods of the same pairs (faithful = no EPY in brood; unfaithful = at least 1 EPY in brood). There was no difference between years ($\chi^2 = 1.43$, d.f. = 1, $P = 0.23$).

of sex and year [logistic regression; β (SE) = –0.13 (0.78), Wald = 0.03, d.f. = 1, $P = 0.87$] or sex [β (SE) = –0.51 (0.34), Wald = 2.25, d.f. = 1, $P = 0.13$], but tended to be influenced by year [both sexes combined; 2001/2002: 22/37 returned; 2002/2003: 47/113 returned; β (SE) = –0.72 (0.39), Wald = 3.51, d.f. = 1, $P = 0.061$]. Males and females showed a high degree of site fidelity, with 92% of returning males ($n = 26$) and 69% of returning females ($n = 13$) occupying the same or adjacent territory as they held in 2002. Ten females with EPP in their brood in 2002 were breeding in 2003, and all were either widowed (i.e. social partner in 2002 was absent in 2003; $n = 7$) or had changed social partner ($n = 3$). For three of these females no previous extra-pair mates returned in 2003, while for the remaining seven females an extra-pair male that sired offspring in her nest in 2002 was still alive in 2003. None of these females chose a previous extra-pair mate as their new social partner. In five cases the identities of the social partners of the extra-pair males were known in both years. All five extra-pair males changed social females in the second year, suggesting that they were available as social partners. Both the male and female of four pairs present in 2002 were also present in 2003; in three cases they changed social partners and in one case they remated with the same social partner. The three females that changed partner were equally unfaithful to their previous and new social partner [mean percentage EPP with previous partner (2002) vs. new partner (2003): 67% vs. 67%]. In the single case where the pair remained together, the male lost paternity of a larger percentage of offspring in 2003 (0% in 2002 vs. 40% in 2003).

Genetic mate choice

Of the 21 females that produced EPY in both their broods within a single season, 65% produced extra-pair offspring sired by different extra-pair males in the second brood than in the first brood, even though the extra-pair sires from the first brood were still alive (2002: 9/14; 2003: 5/7; $\chi^2 = 0.11$, d.f. = 1, $P = 0.74$). The remaining 35% of females produced offspring in their second brood that were sired by at least one of their previous extra-pair partners. Four out of six females that bred both in 2002 and 2003, and for which at least one extra-pair father from 2002 was still alive in 2003, produced extra-pair offspring sired by a different extra-pair mate in 2003.

Males that sired EPY in other nests were less often cuckolded in their own nests than males that did not sire EPY in other nests in 2002, but not in 2003 (Fig. 5). Similar results were found when males from the outer territories were excluded (Mann–Whitney U-test; 2002: $U = 26.0$, $n_{\text{no EPY}} = 9$, $n_{\text{EPY}} = 13$, $P = 0.03$; 2003: $U = 27.5$, $n_{\text{no EPY}} = 8$, $n_{\text{EPY}} = 9$, $P = 0.40$; 2002 + 2003: $U = 62.5$, $n_{\text{no EPY}} = 13$, $n_{\text{EPY}} = 15$, $P = 0.10$). However, after correcting for FDR, this relationship was no longer significant (corrected $\alpha = 0.017$). We found no relationship between the proportion of EPP in a male's own nest and the number of EPY he sired in other nests (Spearman correlation; all males: $P > 0.25$; males from the outer territories excluded: $P > 0.4$).

Pairwise relatedness

The results for 2002 and 2003 were similar in all analyses below; therefore we only present the results of 2002 and 2003 combined. The pairwise relatedness of a female to her social male was not related to the occurrence of cuckoldry (relatedness of pairs with no EPP vs. with EPP: –0.02 ± 0.04 ($n = 16$) vs. –0.03 ± 0.02 ($n = 45$); $t = 0.42$, d.f. = 59, $P = 0.68$)

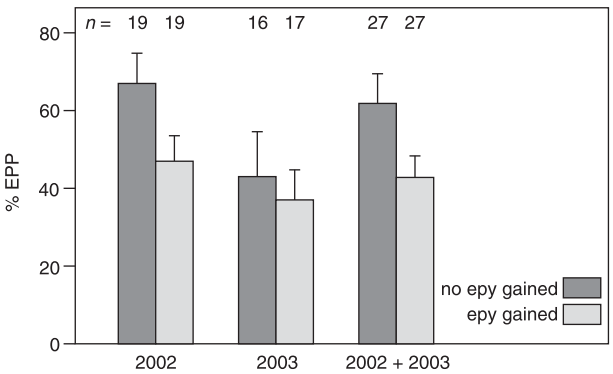

Fig. 5 The percentage of EPP in broods of males that did and did not gain EPY in other broods. Sample sizes are indicated in the figure (Mann–Whitney U-test; 2002: $U = 114.0$, $P = 0.050$; 2003: $U = 130.5$, $P = 0.84$; 2002 + 2003: $U = 260.0$, $P = 0.065$).

nor to the percentage of EPP in a brood ($r_s = 0.13$, $n = 61$, $P = 0.31$). A pairwise comparison showed that extra-pair males did not differ in their relatedness to the female from the social males (extra-pair male vs. social male: –0.01 ± 0.02 vs. –0.04 ± 0.02; $t = -1.04$, d.f. = 46, $P = 0.30$). The percentage of offspring sired in a brood by different males was not proportional to their relatedness to the female (excluding nests with no EPP; $r_s = 0.02$, $n = 98$, $P = 0.84$). When two extra-pair males sired offspring in a brood, there was no indication that more offspring were sired by the less related male than by the more related male, or vice versa (relatedness of extra-pair males with most paternity vs. with least paternity in a brood: 0.002 ± 0.04 vs. 0.002 ± 0.05; $t = 0.004$, d.f. = 10, $n = 1.0$).

Discussion

The high frequency of EPP found in our study population of reed buntings (i.e. 51% of offspring in 74% of broods were sired by extra-pair males) is comparable to that found in a British population (55% of offspring in 86% of broods; Dixon *et al*. 1994); these frequencies of EPP are among the highest found in socially monogamous birds (Griffith *et al*. 2002). We used patterns of paternity in reed bunting broods of both the same and different pairs to test whether these patterns match the specific predictions of four female benefit hypotheses: (i) assessing potential future partners (ii) increasing the genetic diversity of offspring, or increasing the genetic quality of offspring, either by seeking (iii) good genes or (iv) compatible genes. We found that most patterns, but not all, were in support of the latter hypothesis.

Brood predation may influence male and female strategies. If predation of the nest reflects the low quality of the social male, females may increase their extra-pair mating efforts after nest failure (Gissing *et al*. 1998). On the other hand, a male's investment in nest defence may be influenced by high levels of EPP (Weatherhead *et al*. 1994). In reed buntings, females mainly build the nest (Cramp & Perrins 1994; personal observation) and probably decide on its location and concealment, while male nest defence against mammalian predators is unlikely to be successful. In addition, we found no difference in the percentage of EPP between first nests that fledged and that were naturally predated (Bouwman 2005), suggesting that male nest defence is unrelated to percentage of EPP. We therefore expect that predation is independent of the occurrence of EPP.

Assessing potential future partners

High site fidelity and multiple breeding may provide females with the opportunity to select the best available partner based on information gained in the previous nesting attempt (Beletsky & Orians 1991; Weatherhead 1999). A multispecies comparison showed a positive association between EPP levels and divorce rate (Cézilly & Nager 1995). However, in our study no social mate switching was observed within a season; neither between the prebeeding period to the breeding period, nor between breeding attempts when both members of the pair were still present in the study area. Only few males and females belonging to the same pair survived to the subsequent season; of these the majority of individuals paired with a different social mate. When choosing a new social partner (e.g. through mate-switching or though being widowed), females did not form pairs with extra-pair mates from the previous year, even though at least some of these males appeared to be available. These results indicate that individuals do not engage in EPCs in order to test potential future partners, which is in agreement with findings in other species (yellow warbler, *Dendroica petechia* (Yezerinac *et al*. 1995); red-winged blackbird, *Agelaius phoeniceus* (Weatherhead 1999); black-capped chickadee, *Parus atricapillus* (Ramsay *et al*. 2000). In oystercatchers (*Haematopus ostralegus*), where life-long monogamy is the rule, there is some evidence that females use EPCs to test potential future mates; however, these EPCs rarely result in EPFs (Heg *et al*. 1993).

Genetic diversity

Although some authors have made clear predictions concerning the genetic diversity hypothesis (Westneat *et al*. 1990; Kempenaers & Dhondt 1993), others have discarded it as unlikely to be a reason for extra-pair mating behaviour (Birkhead & Møller 1992). Mating with just one male will produce considerable genetic diversity just through meiosis and recombination, and mating with multiple males will not increase this diversity to any great extent (Williams 1975). The patterns of EPP observed in this study were inconsistent with the hypothesis that females seek genetic diversity for their offspring, since (i) EPP was not evenly distributed among broods and (ii) more broods than expected were sired by a single male, either the social male or an extra-pair male.

Good genes vs. genetic compatibility

When distinguishing between the good genes and the genetic compatibility hypotheses, we found that most patterns of EPP were in support of the genetic compatibility hypothesis. First, in contrast to the good genes hypothesis, the genetic compatibility hypothesis allows reciprocal extra-pair paternity between males (Kempenaers & Dhondt 1993), as was found in this study. Second, there is likely to be less variability in male mating success (Kempenaers & Dhondt 1993). We found that males siring EPY in other nests were not more successful at siring offspring in their own nests (after correcting for multiple testing). Neither was there a relationship between the number of EPY a male gained in other nests and the percentage of paternity he gained in his own nests.

Some results appear to be in support of neither the good genes nor the genetic compatibility hypothesis. There was no consistency in the proportion of EPP between nests of the same female within a season in 2002 or, after correcting for multiple testing, in 2003. Furthermore, females were not consistent in choosing the same extra-pair partner for different breeding attempts. If females are choosy and males signal honestly their genetic quality, paternity is expected to be consistent in subsequent broods (Weatherhead 1999). However, females may judge a male's absolute quality through precopulatory mechanisms (direct mate choice), but it is less likely that these mechanisms may be used for judging a male's genetic compatibility. To identify genetically compatible sperm postcopulatory mechanisms (either cryptic female choice or sperm competition) are more likely to be used (Jennions & Petrie 2000). If multiple males are compatible, and females mate with multiple males to allow postcopulatory mechanisms to choose the most compatible sperm, the identity of males siring offspring in subsequent nests may vary. This may provide an explanation for the results found in this study.

We did not find any evidence for the pairwise relatedness of females to either the social or extra-pair male to affect EPP, which is consistent with the findings in a Norwegian population of reed buntings (Kleven & Lifjeld 2005). However, since these data on pairwise relatedness were only based on six and nine microsatellite loci, respectively, we cannot exclude the possibility that genetic compatibility between individuals is based on other loci, such as the major histocompatibility complex genes (Freeman-Gallant *et al*. 2003).

Recent findings in the reed bunting show that older males are more successful at gaining fertilizations in their own broods (Bouwman & Komdeur 2005) and in other broods (Bouwman *et al*. 2006). If females are fertilized by an older male, they may gain good 'viability' genes for their offspring (Trivers 1972; Manning 1985; Kokko & Lindstrom 1996; but see Hansen & Price 1995). The good genes and the genetic compatibility hypotheses need not be mutually exclusive, as females may choose high quality as well as compatible genes (Jennions & Petrie 2000; Puurtinen *et al*. 2005). Female blue tits gained different benefits from different males, as copulations with local males produced offspring with good genes, while copulations with nonlocal males increased offspring heterozygosity (Foerster *et al*. 2003). Alternatively, females may use precopulatory mate choice to choose males above a certain quality threshold (e.g. old males) and after mating use postcopulatory mechanisms to identify genetically compatible sperm (Jennions & Petrie 2000; Thuman & Griffith 2005).

Fitness consequences

In order to reveal underlying mechanisms, it is important to determine differences between maternal and paternal half-sibs (Griffith *et al*. 2002). If females use EPCs to assess potential future partners or seek genetic diversity for their offspring, maternal half-sibs are not expected to be different from each other. If however, females seek good or compatible genes, EPY are expected to be 'fitter' than WPY when comparing maternal half-sibs (Griffith *et al*. 2002). In the case of compatible genes, EPY may be 'fitter' than their paternal half-sibs, raised in the father's own nest (Johnsen *et al*. 2000). Recently we found in the same study population that extra-pair offspring had longer tarsi than their maternal half-siblings, but not than their paternal half-siblings (Bouwman *et al*. 2006). There were no differences in mass, growth rate, condition, immunocompetence or heterozygosity between half-siblings in our study population (Bouwman *et al*. 2006) or in a Norwegian population of reed buntings (Kleven & Lifjeld 2004; Kleven & Lifjeld 2005). Unfortunately, we do not have any long-term data on the survival and reproductive success of within- and extra-pair offspring. Without these data, it remains unclear whether the difference in tarsus length is related to a difference in fitness benefits.

Female control?

The occurrence of EPP is not likely to be the result of only the female deciding whether or not to engage in extra-pair matings, but of the interaction between the female, social male and extra-pair male (Westneat & Stewart 2003). If no EPP is found in a brood, this may be the result of the female not being 'interested' in EPCs, or because she has been prevented from doing so (e.g. by mate guarding behaviour of her social partner). Alternatively, EPCs may not necessarily lead to fertilizations. Due to the difficult nature of gathering data on male–female interactions, we have been unable to distinguish between these possibilities, as has been the case in many studies. Female reed buntings are expected to have at least some control over which males gain copulations, since we never observed any forced copulations in the field, nor were any seen in a captive population of reed buntings (E. Nemeth & K. Wingels, personal communication). Moreover, in the captive population, females were seen to actively solicit EPCs (E. Nemeth & K. Wingels, personal communication). Therefore, we feel that it is justified to assume that the observed patterns of EPP are at least to some extent the result of female choice to engage in EPCs. Nevertheless, we acknowledge that we adopted only the female point of view in this study, and that male behaviour should be taken into account. Male mate guarding behaviour has been shown to be effective in a different population of the same species (Marthinsen *et al*. 2005; but see O'Malley 1993). However, including such data was outside the scope of this study, but we would like to encourage others to investigate these forces that may affect mating outcomes.

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Karen Bouwman (Groningen, the Netherlands) conducted this study as part of her PhD research investigating sexual selection and patterns of extra-pair paternity in the reed bunting. This study was a collaboration between the laboratories of Terry Burke (Sheffield, UK), who is interested in sexual selection and population genetics, and Jan Komdeur (Groningen, the Netherlands), whose interests include sexual selection and sex allocation.
